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LEGIONELLA PNEUMOPHILA TOXIN, ISOLATION AND PURIFICATION

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INTRODUCTION

We are all aware of the dramatic outbreak of what is now termed Legionnaires' Disease that occurred in the city of Philadelphia, Pennsylvania, in the summer of 1976 in which 29 people died. Eight months following that outbreak a gram negative bacteria now termed Legionella pneumophila was isolated and identified as the etiologic agent by Drs. Fraser (1) and McDade (2).

Although Legionnaires' Disease has been recognized in 43 states and the District of Columbia (3) the disease is not restricted to the North American continent. In addition to Canada, cases have been reported in Australia, England, Israel, Scotland, Denmark, Spain and The Netherlands. The largest outbreak of legionellosis yet documented outside the United States occurred in Vasteras, Sweden from August 28 to September 21, 1979, and involved 67 cases (4).

We now recognize two distinct clinical syndromes associated with this organism. The first termed Legionnaires' Disease, involves patients ranging in age from 3 to 82 years. The symptoms include headaches, myalgia, and general malaise. Within one or two days there is a rapid temperature rise associated with chills. A moderate nonproductive cough is common. The symptoms then progress to include chest pain, abdominal pain, vomiting and mental confusion. Diarrhea is seen in one sixth of the patients. The disease is basically a rapidly progressive and fulminant pneumonia with chest X-rays initially showing patchy infiltrates that may have an interstitial or consolidated appearance which typically in the untreated case progress to nodular consolidation that may be unilateral or bilateral. The pneumonic form of the disease has a 16 percent lethality in normal individuals and a 54 percent lethality in immunologically compromised patients. Death is associated either with respiratory failure or shock.

The second clinical syndrome presents as a non-pneumonic, non-lethal debilitating flu like illness popularly termed "Pontiac Fever" named after the city in Michigan where the original outbreak occurred. Fraser (5) has pointed out that what determines whether L. pneumophila will cause Legionnaires' Disease or Pontiac Fever is entirely unknown. He did suggest that Pontiac Fever might result from a large dose of nontoxigenic organisms. At the time he made that suggestion no known toxins were recognized in any isolated strains of

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L. pneumophila. However, in 1978 Dr. Harvey Friedman (6) and Dr. Washington Winn (7) both postulated that a toxin or toxins might be involved in the pathogenesis of Legionnaires' Disease for two dissimilar reasons. Dr. Friedman, a clinician, noted that the disease generally involved the lungs, kidneys and central nervous system. Although the pathogenesis of the multiple organ involvement was unclear he offered as a possible explanation the production of a toxin by L. pneumophila. Dr. Winn, a pathologist, reviewed 14 fatal cases from the 1977 Legionnaires' Disease outbreak in Vermont and suggested that the production of a cytolytic toxin by L. pneumophila could explain the lysis of inflammatory exudate cells and the infarct like necrosis of alveolar walls that were seen in several cases. Our own interest in the possible toxin or toxins of L. pneumophila extends back to the concepts of Friedman and Winn but had its impetus in our own reported findings that AKR/J mice challenged with lethal doses of L. pneumophila organisms displayed a histologic pattern of randomly scattered hepatocellular necrosis involving individual cells along with larger foci of coagulative necrosis and a scattering of hepatocytes with coarse vesiculated cytoplasm compatible with the effects caused by a toxin (8).

This paper reports the first identification of a low molecular weight protein toxin derived from a cell-free extract of sonicated L. pneumophila which displays an *in vivo* lethality. The purification procedures involve acid precipitation, gel chromatography, and preparative isotachopheresis.

The antigenic relationship of this Legionella toxin with a similar extract obtained from a newly discovered, but genetically unrelated gram negative rod; popularly termed the Pittsburgh Pneumonia Agent, is also demonstrated.

MATERIALS AND METHODS

Toxin production

The Atlanta, Chicago and Washington Strains of L. pneumophila kindly supplied by the Center for Disease Control (Atlanta, Georgia) were streaked on modified Mueller-Hinton agar and incubated at 35°C in 5% CO₂ for four days. Cells from surface growth were harvested, washed and centrifuged three times in normal saline. 10 gram aliquots of the washed pellets were then sonicated at 5°C three times for two minute intervals using a Fisher Sonic Dismembrator Model 300. The disrupted cells were resuspended in a total volume of 40 ml of normal saline and centrifuged at 1500 X g for 30 minutes with the use of a Sorvall RC-2B refrigerated centrifuge at 5°C. The supernatant material was first filtered through 0.45 µm membrane filter and then through a 0.22 µm filter. The toxicity of each crude supernatant preparation was measured by its ability to kill AKR/J mice by intraperitoneal inoculation within a 24 hour period.

Molecular weight determination by gel filtration

A Sephadex G 50 column (1.5 X 45.0 cm) was calibrated according to the method of Andrews (9) with the use of Chymotrypsinogen A, Ribonuclease A, and Apoprotinin as markers.

Preparation of antiserum

One milligram amounts of protein from Legionella acid supernatant was emulsified in one milliliter of incomplete Freund's adjuvant and injected either intramuscularly into goat or into the foot pads of rabbits. The animals were boosted with the same material after 28 days and 7 days later bled for antibodies. Pre bleed serum had been obtained as a control.

RESULTS

Toxin isolation

Step 1: Acid precipitation of crude toxin. 1.0 N HCl acid was slowly added to rapidly stirred crude toxin until pH 3.5 was obtained. This opalescent mixture was then recentrifuged at 1500 X g as previously noted. After membrane filtration the toxic activity was found to be localized to the acid supernatant and the pellet was discarded. This step resulted in approximately a 70% reduction in total protein.

Step 2: Bio Gel A 5m gel filtration. 10 ml aliquots of acid supernatant from the previous step were applied to a 5.0 X 11.0 cm column of Bio Gel A 5m and eluted with 0.1 M phosphate buffered saline pH 7.4. The flow rate did not exceed 1 ml per hour per cm² of column cross sectional area. 12 ml fractions were collected. The toxic activity appears with the void volume. The first peak constituted approximately 5 to 10% of the total protein applied to the A 5m column depending upon the individual acid supernatant preparation. There were no observable differences in lethality between toxin derived from any of the three Legionella strains (Atlanta, Chicago or Washington). See Fig. 1.

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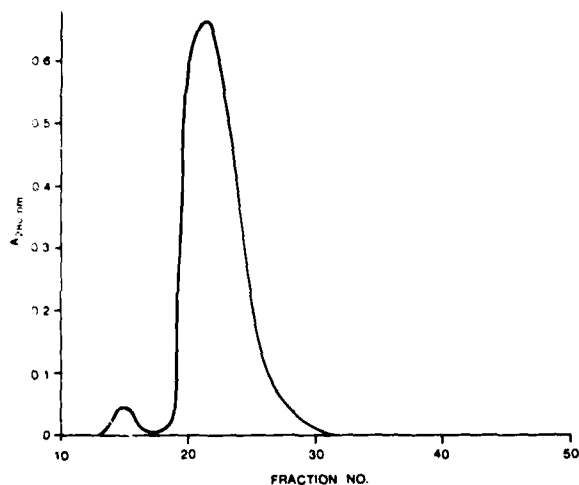


Fig. 1. Elution pattern of *Legionella* acid supernatant toxin preparation on a Bio Gel A 5m column (5 X 11 cm) equilibrated with 0.1 M PBS. The sample volumes were approximately 10 ml. 12 ml fractions were collected. The toxin activities were determined by intraperitoneal inoculation into AKR/J mice.

Step 3: Preparative isotachopheresis. Preparative isotachopheresis was performed using a vertical column electrophoresis apparatus (LKB 7900 Uniphor). The buffers employed and the 4.5% polyacrylamide gel supporting medium were made according to the method of Svendsen and Rose (10). Single buffered gel columns (cross sectional area 3.48 cm², length 14 cm) were used and the Ampholine carrier (pH 4 to 9) was mixed with the sample. Deaerated tris-phosphate, pH 7.05, was used both in the lower electrode (anode) chamber and the elution buffer. The terminating buffer, Tris-e-aminocaproate (Tris EACA), pH 8.45, was used in the upper electrode (cathode) chamber and in the column above the gel and sample.

A sample of 7-10 ml of the first A 5m acid supernatant peak containing 50 µg of protein per ml was mixed with 1.0 ml of glycerol to increase the viscosity. The mixture was then layered on top of the gel by tubing inserted through the top layer of Tris-EACA buffer which was held within a few mm of

the gel surface. The experiments were performed using a constant power source (LKB 2127). A current of 4 mA and a starting voltage of 1.5 KV was applied in every run. Cooling water was ambient which usually ranged between 20-28°C.

The elution rate was 18 ml/hr. The eluate was recovered in 12 ml aliquots on a fraction collector. UV absorption at 280 nm of each fraction was read on a Beckman DBG spectrophotometer. See Figure 2.

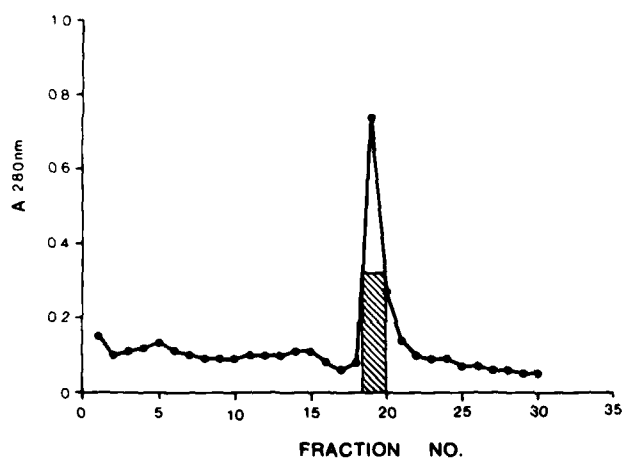


Fig. 2. Preparative isotachopheresis run from Uniphor column in which a semi-purified Legionella toxin was separated according to Svendsen and Rose (10). The cross hatched area denotes lethality activity for AKR/J mice.

Molecular weight. The toxin eluted as one symmetrical peak. The molecular weight of the toxic moiety isolated by preparative isotachopheresis was estimated by measuring the elution volume on a column of Sephadex G 50 and found to be 3400 daltons. See Fig. 3.

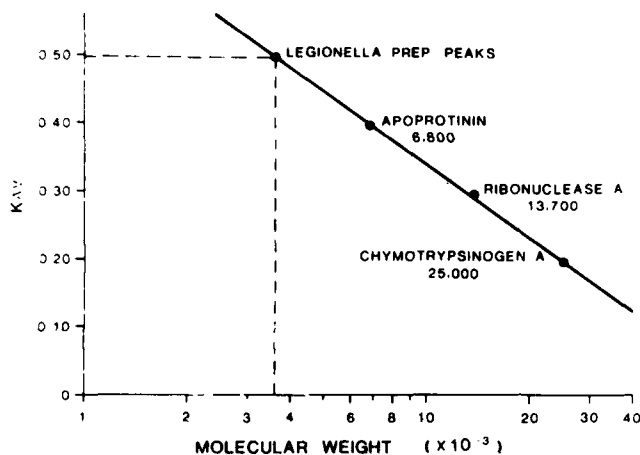


Fig. 3. Determination of Legionella toxin on Sephadex G 50 column (1.5 X 45.0 cm) by extrapolation.

Immunological reactivity of preparative isotachopheresis derived toxin.
The relationship of Legionella acid supernatant and the preparative isotachophore peak is best depicted by Fig. 4.

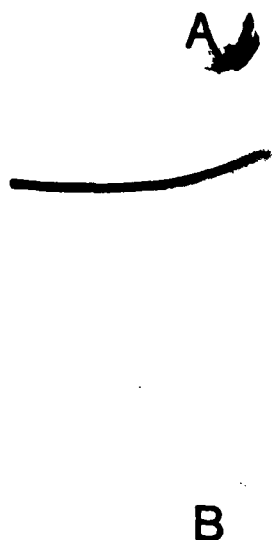


Fig. 4. Legionella toxin examined by immunoelectrophoresis. Antigen in well A is Legionella acid supernatant. In the well B is the toxin; the preparative isotachophore peak of acid supernatant. Anode to the left. The rabbit antibodies in the central trough were directed against Legionella acid supernatant. A Coomassie brilliant blue R stain was used.

A single precipitin band identifies the Legionella preparative isotachophoresis peak. A single peak on G 50 gel filtration and preparative isotachophoresis coupled with a single precipitin band on immunoelectrophoresis are suggestive of a fair degree of purity.

Antigenic relationship of Legionella toxin and a similar protein derived from the Pittsburgh Pneumonia Agent

This section constitutes an addendum and is included here to highlight a relationship between organisms which are genetically unrelated. Recently Pasculle (11) and Myerowitz (12) reported the first isolation of a new gram negative bacteria called the "Pittsburgh Pneumonia Agent" (PPA). PPA resembles L. pneumophila in pathogenicity, growth requirements and composition of fatty acids. The two bacteria differ in their genetic relatedness, reported antigenic composition and colonial morphology. Using the identical techniques we have just described on extracts of L. pneumophila and PPA we have been recently

able to show that PPA acid supernatant preparations and preparative isotachophore peaks are also able to kill AKR/J mice by intraperitoneal inoculation. Immunodiffusion studies show that Legionella acid supernatant and toxin share a common antigen with PPA acid supernatant and its preparative tachophore peak which has the same molecular weight (3400 daltons) as the Legionella toxin. Parenthetically it should be noted that Legionella lipopolysaccharide (endotoxin) is functionally, biochemically and antigenically unrelated to this low molecular weight entity. See Fig. 5.

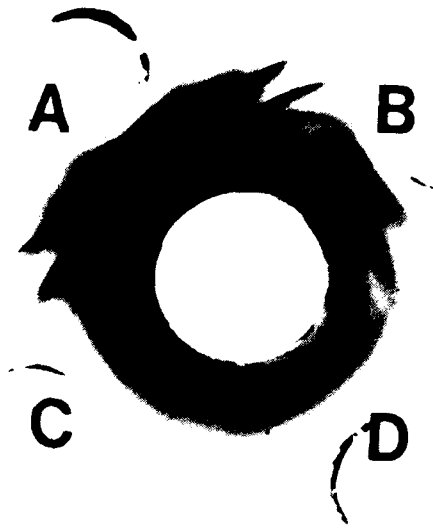


Fig. 5. Immunodiffusion plate well A and B contain Legionella acid supernatant and toxin (preparative isotachophore peak) respectively, wells C and D contain PPA acid supernatant and PPA preparative isotachophore peak. The center well contains antiserum to Legionella acid supernatant. A line of identity joins a common antigen in the four outer wells.

DISCUSSION

The presence of a toxin associated with L. pneumophila has long been suspected. In this paper we have demonstrated a 3400 M.W. intracellular protein which is lethal when injected intraperitoneally into AKR/J mice. The techniques used in the isolation of this toxin included acid precipitation, gel filtration, and preparative isotachophoresis; all of which are now fairly

standard. The advantages that the preparative isotachopheresis step conferred were that it allowed the dissociation of a high molecular weight protein entity (5,000,000 M.W.) and that it organized the toxic activity under a single low molecular weight protein peak. Legionnaires' Disease and Pittsburgh Pneumonia are clinically similar but are caused by genetically unrelated gram negative bacteria. The fact that we can show that both extracts are toxic in vivo and antigenetically related opens up intriguing possibilities in understanding the pathogenesis of these two previously unrecognized bacterial agents.

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